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PTO/SB/05 (4/98)
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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No.	44894
First Inventor or Application Identifier	PRASAD, Kailash
Title	ANTIOXIDANT ACTIVITY IN SDG METABOLITES
Express Mail Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: **Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231**

JC829 U.S. PTO 09/599662 06/23/00

1. [X] * Fee Transmittal Form (e.g., PTO/SB/17) *(Submit an original and a duplicate for fee processing)*
2. [X] Specification [Total Pages 12] *(preferred arrangement set forth below)*
   - Descriptive title of the Invention
   - Cross References to Related Applications
   - Statement Regarding Fed sponsored R & D
   - Reference to Microfiche Appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings (*if filed*)
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure
3. [X] Drawing(s) (35 U.S.C. 113) [Total Sheets 8] (Informal)
4. Oath or Declaration [Total Pages]
   - a. [X] Newly executed (original or copy)
   - b. [ ] Copy from a prior application (37 C.F.R. § 1.63(d)) *(for continuation/divisional with Box 16 completed)*
     - i. [ ] DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).

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5. [ ] Microfiche Computer Program (*Appendix*)
6. Nucleotide and/or Amino Acid Sequence Submission (*if applicable, all necessary*)
   - a. [ ] Computer Readable Copy
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ACCOMPANYING APPLICATION PARTS

7. [X] Assignment Papers (cover sheet & document(s))
8. [ ] 37 C.F.R.§3.73(b) Statement (*when there is an assignee*) [ ] Power of Attorney
9. [ ] English Translation Document (*if applicable*)
10. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
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12. [ ] Return Receipt Postcard (MPEP 503) (*Should be specifically itemized*)
13. [X] * Small Entity Statement(s) (PTO/SB/09-12) [ ] Statement filed in prior application, Status still proper and desired
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[ ] Customer Number or Bar Code Label (Insert Customer No. or Attach bar code label here) or [ ] Correspondence address below

Name	Norris M. Eades				
Address	KIRBY EADES GALE BAKER				
	P.O. Box 3432, Station D				
City	Ottawa	State	Ontario	Zip Code	K1P 6N9
Country	CANADA	Telephone	(613) 237-6900	Fax	(613) 237-0045

Name (Print/Type)	Norris M. Eades	Registration No. (Attorney/Agent)	25,263
Signature	[signature]	Date	June 22, 2000

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Docket Number (Optional): 44894

Applicant, Patentee, or Identifier: PRASAD, Kailash
Application or Patent No.:
Filed or Issued:
Title: ANTIOXIDANT ACTIVITY IN SDG METABOLITES

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TITLE IN ORGANIZATION OF PERSON SIGNING President & CEO
ADDRESS OF PERSON SIGNING 304 Kirk Hall, 117 Science Place, Saskatoon, SK
SIGNATURE B. F. Peterman DATE June 12, 2000

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Cross-Reference to Related Application

This application claims the benefit of U.S. Provisional Application No. 60/141,254, filed June 30, 1999.

Background of the Invention

This invention relates to a method for the use of metabolites of secoisolariciresinol diglucoside (SDG) for the treatment of diseases or conditions requiring administration of an antioxidant. These metabolites include secoisolariciresinol (SECO), enterodiol (ED) and enterolactone (EL).

Reactive oxygen species, which include superoxide anion ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$) and singlet oxygen ($^1O_2$), have been implicated in the pathophysiology of numerous diseases, including hypercholesterolemic atherosclerosis, diabetes mellitus, ischemic/reperfusion injury, volume or pressure overload heart failure, hemorrhagic shock, endotoxic shock, ageing, inflammatory bowel disease (Crohn's disease, ulcerative colitis), Parkinson's disease, rheumatoid arthritis and stroke.

Antioxidants such as vitamin E, secoisolariciresinol diglucoside (SDG), probucol, vitamin C, superoxide dismutase, catalase, sulphasalazine, and various other drugs without antioxidant activity, have been shown to be effective to a varying degree in the diseases referred to above. These drugs, with the exception of vitamin C and E and SDG, are expensive and have adverse side effects.

As described in Prasad, U.S. Patent 5,846,944, SDG, isolated from flaxseed, has been shown to be effective in lowering cholesterol, and in reducing the development of atherosclerosis in hypercholesterolemic rabbits. It is also effective in reducing the incidence of diabetes mellitus and preventing endotoxic shock.

Summary of the Invention

Reactive oxygen species are known to be involved in the pathophysiology of ageing and numerous diseases, such as hypercholesterolemic atherosclerosis, type I and

type II diabetes, ischemic heart disease, heart failure, endotoxic and hemmorhagic shock, inflammatory bowel disease, rheumatoid arthritis, Parkinson's disease, and stroke.

Secoisolariciresinol diglucoside (SDG), which is obtained from flaxseed, is metabolized to secoisolariciresinol (SECO), enterodiol (ED), and enterolactone (EL). A description of the above metabolites can be found in a report by R.K. Harris et al. (1991) Methods Development for Phytochemical Compliance Markers in Designer Foods (Flaxseed Powder), Midwest Research Institute. These metabolites are respectively 4.86, 5.02, and 4.35 times more potent than vitamin E, and 3.82, 3.95, and 3.43 times more potent than SDG. Vitamin E, SDG and various other drugs, some with antioxidant activity and some without, are currently used for the treatment of the above diseases.

Drugs presently used to treat the diseases listed above, are expensive and have been less than satisfactory for the treatment of these diseases because of their adverse side effects. The discovery of SDG metabolites offers a safe, less expensive antioxidant that is useful in the treatment of these diseases and conditions. They are derived from dietary flaxseed and are therefore from a natural source, having little to no side effects.

Thus, the present invention relates to the use of secoisolariciresinol (SECO), enterodiol (ED) or enterolactone (EL) for the treatment of diseases or conditions requiring administration of an antioxidant. These diseases or conditions include hypercholesterolemic atherosclerosis, type I and type II diabetes, ischemic heart disease, heart failure, endotoxic and hemmorhagic shock, inflammatory bowel disease, rheumatoid arthritis, Parkinson's disease, and stroke.

The SECO, ED or EL is preferably used in purified form and can be administered orally or intravenously. It can, for instance, be administered in a once daily oral dosage of about 5-15 mg per kg of body weight. The oral doses may conveniently be in the form of tablets or capsules and these metabolites may be used together with a variety of pharmaceutically acceptable diluents or carriers.

Morbidity and mortality associated with the diseases referred to above and their complications, such as lost wages, increased health costs and social burdens, are enormous. Treatment with the metabolites according to this invention serve to reduce or prevent the late complications associated with these diseases. The morbidity and mortality associated with these diseases is reduced or prevented. This reduces the burden

of illness to society, and overall health care costs, and permit these patients to return to the workplace and be productive members of society.

Brief Description of the Drawings

In the drawings that illustrate the present invention:

Fig. 1 is representative tracings showing changes in the chemiluminescence (CL) of zymosan-stimulated polymorphonuclear leukocytes chemiluminescence (PMNL-CL) in the (1) absence of and in the presence of 2.5 mg/ml of SDG (2), SECO (3), EL (4) or ED (5).

Fig. 2 is a bar graph showing changes in the integrated CL of unstimulated blood (BL) or zymosan-stimulated blood in the absence of or in the presence of SDG, SECO, ED, EL or Vitamin E [α-tochopherol (α-TP)], each in a concentration of 2.5 mg/ml.

Fig. 3 is a bar graph showing the percent inhibition of PMNL-CL by SDG, SECO, ED, EL and α-TP in similar concentration (2.5 mg/ml).

Fig. 4 is a bar graph showing the effects of various concentrations of SDG on zymosan-stimulated PMNL-CL.

Fig. 5 is a bar graph showing the effects of various concentrations of SECO on zymosan-stimulated PMNL-CL.

Fig. 6 is a bar graph showing the effects of various concentrations of ED on zymosan-stimulated PMNL-CL.

Fig. 7 is a bar graph showing the effects of various concentrations of EL on zymosan-stimulated PMNL-CL.

Fig. 8 is a bar graph showing the effects of various concentrations of α-TP on zymosan-stimulated PMNL-CL.

Description of the Preferred Embodiments

Measurement of Antioxidant Activity

Antioxidant activity of SDG, SECO, ED, EL and vitamin E (alpha-tochopherol phosphate) was measured using the ability of these compounds to reduce the chemiluminescence of activated PMNLs [polymorphonuclear leukocytes

chemiluminescence (PMNL-CL)]. Activated polymorphonuclear leukocytes produce superoxide anion ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and singlet oxygen ($^1O_2$). Chemiluminescence is amplified by luminol, which is converted to an excited aminophthalate ion in the presence of oxidizing species like $O_2^{\cdot}$, H_2O_2, •OH and $^1O_2$. Luminol-dependent CL reflects the amount of activated oxygen species generated from activated phagocytes, thus, this method can be used to monitor the reactive oxygen species produced by PMNLs. Agents which scavenge $O_2^{\cdot}$, H_2O_2, •OH and $^1O_2$ would reduce PMNL-CL. The SDG, SECO, ED and EL were all obtained from Agriculture and Agri-Food Canada, Saskatoon, Saskatchewan.

Venous blood from healthy subjects, after their informed consent, was collected in ethylenediamine tetraacetic acid (EDTA)-containing tubes for PMNL counts, and PMNL-CL. PMNL and WBC counts were made using Technicon H6000 system (Technicon Instruments, Tarrytown, NY). PMNL-CL a measure of reactive oxygen species produced by PMNLs was measured by a method described in Prasad et al., Effect of polymorphonuclear leukocyte-derived oxygen free radicals and hypochlorous acid or cardiac function and some biochemical parameters, Am. Heart J. 119:538-550, 1990. Blood (0.05 ml) was added to a glass tube containing Hank's balanced salt solution (HBSS) buffer (pH 7.40) and luminol at a final concentration of 10^{-4} M. To assess the ability of various test materials in the varying amounts (1.0, 2.5, 5.0 and 10 mg/ml) in the powder form, were each added to the test tube containing blood, shaken well and incubated for 15 minutes at room temperature. The final volume of the mixture in these tubes was 0.5 ml. All the test tubes were placed in a luminometer for 5 min at 37°C, and phagocytosis was initiated by the addition of 0.1 ml (10 mg/ml) of opsonized zymosan prepared by previously described method (Prasad et al., 1990). The chemiluminescence was monitored with an Auto Lumat, LB953 luminometer (Egg Berthold, Berthold Analytical Instrument Inc., 472 Amherst Street, Nashua, NH, 03063) for 3 seconds every 2 or 3 minutes (depending on the sample number) for a period of 60 minutes. The integrated area under the curve gives the total luminal-dependent chemiluminescent response during the period of monitoring, which represents the oxygen derived CL. The difference in the integrated area under zymosan-activated in the absence and in the presence of various compounds under investigation is designated as particular compound

inhibitable oxygen derived radical CL. The unit for chemiluminescence is in counts per minute (cpm). The integrated area under the curve is in cpm·m. The unit for chemiluminescence is cpm·m·10^{-6} PMNLs because the chemiluminescence is expressed in terms of 10^{-6} PMNL counts. The peak chemiluminescence is cpm·10^{-6} PMNLs.

Statistical Analysis

The results are expressed as mean ± SE and n = sample size. One-way Analysis of Variance (ANOVA) followed by Scheffe test was used to derive differences between different groups. A "p" value of less than 0.05 was considered significant.

Comparison of the Antioxidant Activity

A typical tracing of the chemiluminescence (CL) of zymosan-activated PMNLs in blood in the absence or presence of SDG, SECO, EL or ED is shown in Fig. 1. The chemiluminescent activity of PMNLs increased rapidly with the addition of zymosan and reached a peak value within 8-10 min. After its peak, it decreased slowly for the duration of the observation period to reach at prestimulated value at the end of 60 min. SDG, SECO, EL, ED and vitamin E each in the concentration of 2.5 mg/ml decreased chemiluminescent activity of zymosan stimulated PMNLs to varying degrees. The results of the effects of SDG, SECO, EL, ED and vitamin E on the integrated-CL of unstimulated- or zymosan-stimulated blood are summarized in Table I and Fig. 2. There was an increase of approximately 85 folds in the integrated CL with zymosan in the untreated blood. SDG, SECO, EL and ED produced a reduction in CL by 39%, 76%, 48% and 73% respectively in the unstimulated blood (Table I). The percentage inhibition of PMNL-CL with SDG, SECO, ED, EL and vitamin E (α-TP) are shown in Fig. 3. SDG and vitamin E in the concentration of 2.5 mg/ml produced inhibition to similar extent (23.87% vs. 18.75%). SECO and ED produced similar inhibition (91.2% vs. 94.22%). The inhibition produced by EL was 81.57%. The antioxidant activity was highest with SECO and ED and lowest with SDG and vitamin E. The order of antioxidant potency was SECO = ED > EL > SDG = vit. E. The antioxidant potency of SECO, ED, EL and SDG was 4.86, 5.02, 4.35, 1.27 respectively as compared to vitamin E.

Table 1. Integrated CL ($x10^6$ cpm·m·10^{-6} PMNLs) of unstimulated blood (BL) in the absence and presence of SDG, SECO, EL or ED

BL 4.038 ± 0.143	BL + SDG 2.463 ± 0.159* (39)
BL 3.654 ± 0.346	BL + SECO 0.878 ± 0.023* (76)
BL 4.168 ± 0.499	BL + EL 2.177 ± 0.138* (48)
BL 4.038 ± 0.143	BL + ED 1.073 ± 0.048* (73)

The results are expressed as mean ± SE from 16 samples each. BL, blood; SDG, secoisolariciresinol diglucoside; SECO, secoisolariciresinol; EL, enterolactone; ED, enterodiol. The numbers in the bracket show the percent reduction of CL by the particular compound.

*$P<0.05$, BL vs. BL + SDG, BL + SECO, BL + EL, or BL + ED.

Concentration-dependent Response

The effects of 1.0, 2.5, 5.0 and 10.0 mg/ml of SDG, SECO, ED, EL and vitamin E on the zymosan-stimulated PMNL-CL were investigated to determine if the antioxidant activity was concentration-dependent. Effects of various concentrations of SDG on zymosan-stimulated PMNL-CL are shown in Fig. 4. Zymosan in the absence of SDG produced a marked increase in the PMNL-CL. SDG produced a concentration-dependent inhibition of PMNL-CL, the inhibition being 24% with 1.0 mg/ml, 30% with 2.5 mg/ml and 48% with 5.0 mg/ml.

Effects of various concentrations of SECO are summarized in Fig. 5. Zymosan in the absence of SECO produced a marked increase in the PMNL-CL. SECO in the concentration of 1.0, 2.5, 5.0 and 10.0 mg/ml produced an inhibition of zymosan-stimulated PMNL-CL by 78%, 93%, 99.5% and 100% respectively. It appears that 5.0 mg/ml almost completely inhibited the PMNL-CL.

Effects of various concentrations of ED on zymosan-stimulated PMNL-CL are shown in Fig. 6. Zymosan in the absence of ED produced a significant increase in the PMNL-CL. ED inhibited the PMNL-CL by 82%, 96% and 95% respectively in the concentration of 1.0, 2.5 and 5.0 mg/ml. The concentration of 2.5 and 5.0 mg/ml has similar effects.

Effects of various concentrations of EL on the zymosan-stimulated PMNL-CL are summarized in Fig. 7. In the concentration of 1.0, 2.5 and 5.0 mg/ml, it produced an inhibition of PMNL-CL by 81%, 86% and 83% respectively. It appears that maximum effect is obtained with 1.0 mg/ml of EL.

Effects of various concentrations of α-tochopherol (α-TP) on the zymosan-stimulated PMNL-CL are shown in Fig. 8. In the concentration of 1.0 mg/ml, it produced an increase in the PMNL-CL. However in the concentrations of 2.5, 5.0 and 10.0 mg/ml it produced an inhibition of 17%, 88% and 97% respectively. It appears that in small concentrations it stimulates PMNLs.

These results indicate that SDG, SECO, ED, and EL are scavengers of $O_2^{\cdot}$, H_2O_2, •OH and $^1O_2$ and are therefore antioxidants.

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The disclosures of the above articles are specifically incorporated herein by reference.

Claims

1. A method of treatment of a disease or a condition requiring the administration of an antioxidant, which comprises administering to a patient an effective amount of a secoisolariciresinol diglucoside (SDG) metabolite selected from the group consisting of secoisolariciresinol (SECO) in a substantially pure form, enterodiol (ED) in a substantially pure form, and enterolactone (EL) in a substantially pure form.
2. A method according to claim 1 wherein said disease is hypercholesterolemic atherosclerosis.
3. A method according to claim 1 wherein said disease is diabetes type I or type II.
4. A method according to claim 1 wherein said condition is ischemic heart disease.
5. A method according to claim 1 wherein said condition is volume or pressure overload heart failure.
6. A method according to claim 1 wherein said condition is the prevention of myocardial injury during open heart surgery.
7. A method according to claim 1 wherein said condition is the prevention of restenosis following persutaneous transluminal coronary angioplasty (PTCA).
8. A method according to claim 1 wherein said condition is hemorrhagic or endotoxic shock.
9. A method according to claim 1 wherein said condition is ageing.
10. A method according to claim 1 wherein said disease is inflammatory bowel disease (Crohn's disease, ulcerative colitis).
11. A method according to claim 1 wherein said disease is Parkinson's disease.
12. A method according to claim 1 wherein said disease is rheumatoid arthritis.
13. A method according to claim 1 wherein said disease is stroke.
14. A method according to claim 1 wherein secoisolariciresinol diglucoside (SDG) is obtained from flaxseed, and said metabolite is obtained from SDG.

Abstract

The compounds secoisolariciresinol (SECO), enterodiol (ED) and enterolactone (EL), which are metabolites of secoisolariciresinol diglucoside obtained from flaxseed, are used for the treatment of diseases or conditions requiring administration of an antioxidant. Diseases or conditions that may be treated include hypercholesterolemic atherosclerosis, type I and type II diabetes, ischemic heart disease, heart failure, endotoxic and hemmorhagic shock, inflammatory bowel disease, rheumatoid arthritis, Parkinson's disease, and stroke.

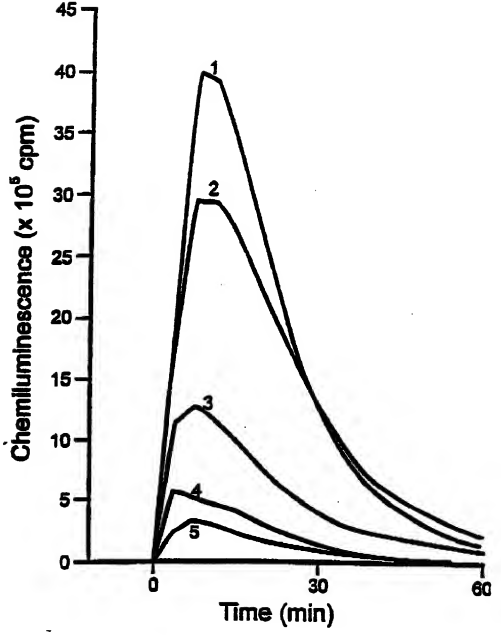
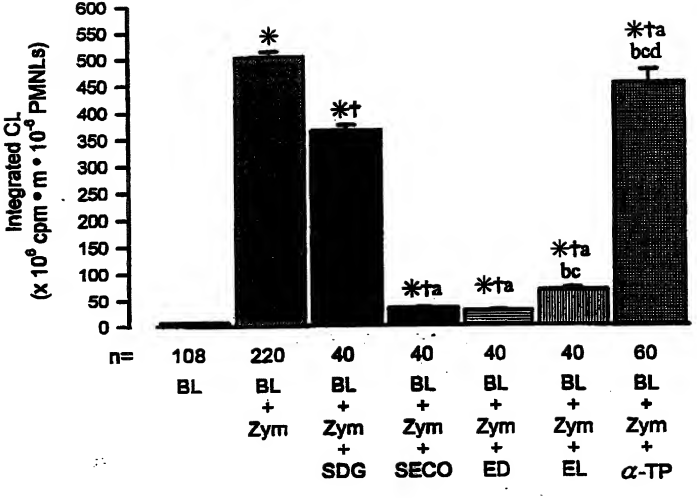
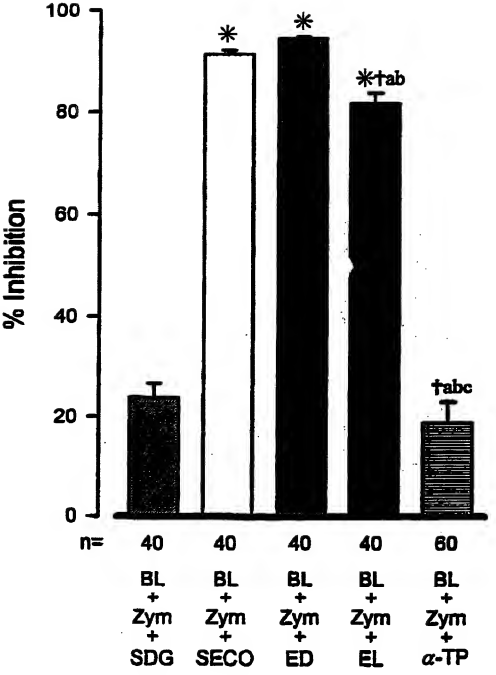

45
40
35
30
25
20
15
10
5
0
Chemiluminescence (x 10^5 cpm)
1
2
3
4
5
0
30
60
Time (min)

FIG. 1



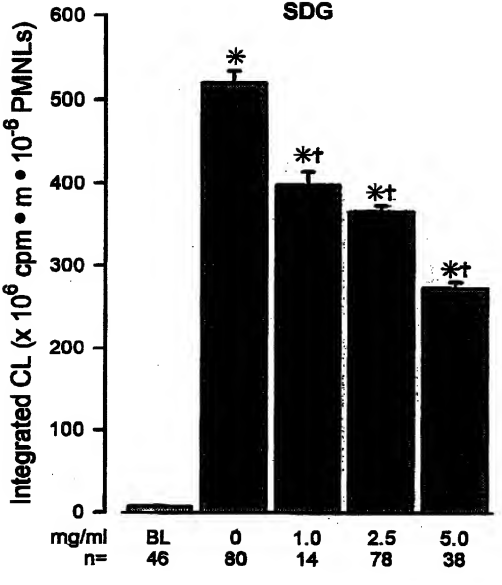
*P<0.05, BL vs others;
†P<0.05, BL + zym vs others;
aP<0.05, SDG vs SECO, ED, EL or α-TP;
bP<0.05, SECO vs ED, EL or α-TP;
cP<0.05, ED vs EL or α-TP;
dP<0.05, EL vs α-TP.

FIG. 2



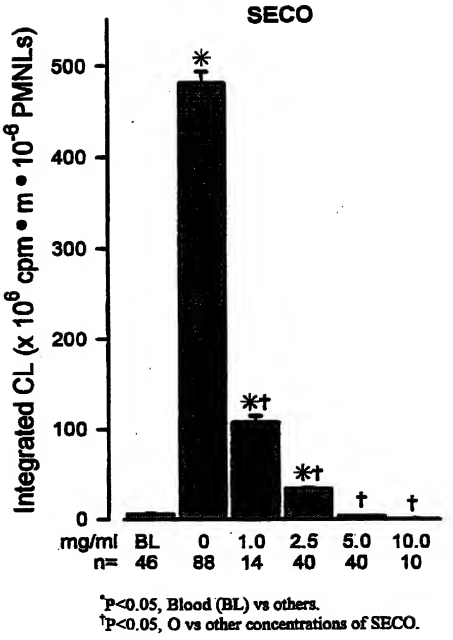
*P<0.05, SDG vs others.
[a]P<0.05, SECO vs ED, EL or α-TP;
[b]P<0.05, ED vs EL or α-TP;
[c]P<0.05, EL vs α-TP

FIG. 3



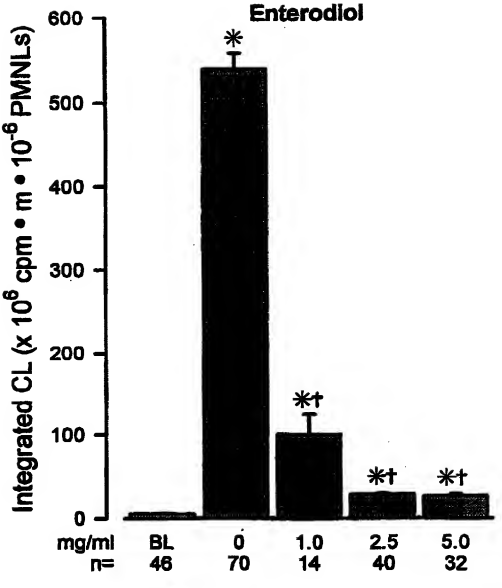
*P<0.05, Blood (BL) vs others.
†P<0.05, O vs other concentrations of SDG.

FIG. 4



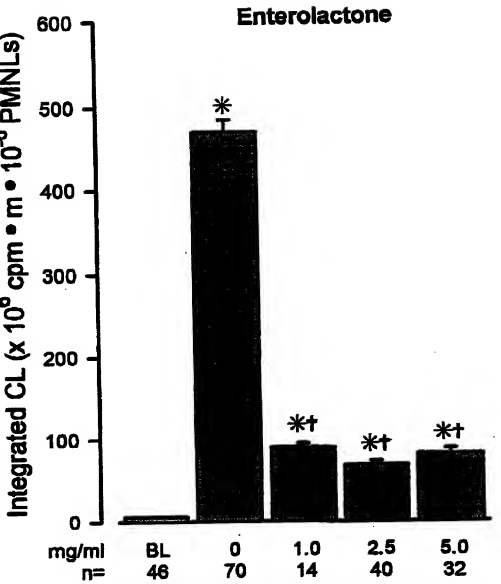
*P<0.05, Blood (BL) vs others.
†P<0.05, O vs other concentrations of SECO.

FIG. 5



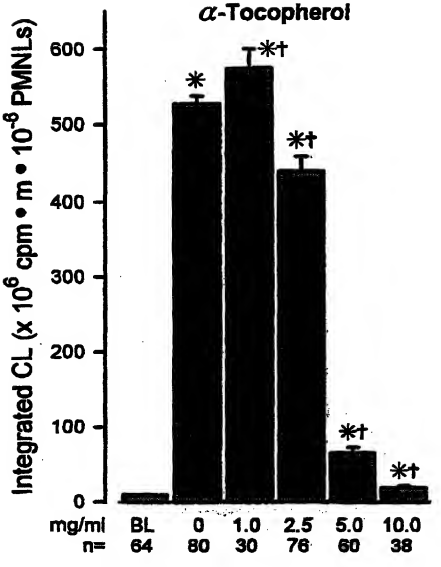
*P<0.05, Blood (BL) vs others.
†P<0.05, O vs other concentrations of ED.

FIG. 6



*P<0.05, Blood (BL) vs others.
†P<0.05, P<0.05, O vs other concentrations of EL.

FIG. 7



*P<0.05, Blood (BL) vs others.
†P<0.05, 0 vs other concentrations of α-tocopherol.

FIG. 8

Please type a plus sign (+) inside this box → [+]

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

■ Declaration Submitted with Initial Filing OR ☐ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	44894
First Named Inventor	PRASAD, Kailash
COMPLETE IF KNOWN	
Application Number	/
Filing Date	
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ANTIOXIDANT ACTIVITY IN SDG METABOLITES

(Title of the Invention)

the specification of which
■ is attached hereto
OR
☐ was filed on (MM/DD/YYYY) [ ] as United States Application Number or PCT International Application Number [ ] and was amended on (MM/DD/YYYY) [ ] (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	
60/141,254	06/30/1999	☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

Please type a plus sign (+) inside this box → [+]

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DECLARATION — Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: ☐ Customer Number OR ■ Registered practitioner(s) name/registration number listed below → Place Customer Number Bar Code Label here

Name	Registration Number	Name	Registration Number
Norris M. Eades	25,263	Kimberley A. Lachaine	33,319
Edwin J. Gale	28,584	Robert K. Feutlinske	37,994
John A. Baker	26,656	Andrew J. Bauer-Moore	44,449

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to: ☐ Customer Number or Bar Code Label [ ] OR ■ Correspondence address below

Name	NORRIS M. EADES,						
Address	KIRBY, EADES, GALE, BAKER,						
Address	P.O. Box 3432, Station D,						
City	Ottawa	State	Ontario	ZIP	K1P 6N9		
Country	CANADA	Telephone	(613)237-6900	Fax	(613)237-0045		

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
Kailash	Prasad

Inventor's Signature	[signature]					Date	June 8, 2000
Residence: City	Saskatoon	State	Saskatchewan	Country	Canada	Citizenship	CA
Post Office Address	358 Assiniboine Drive						
Post Office Address							
City	Saskatoon	State	Saskatchewan	ZIP	S7K 3Z2	Country	Canada

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